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(54) Title: UPREGULATION OF TYPE III ENDOTHELIAL CELL NITRIC OXIDE SYNTHASE BY HMG-CoA REDUCTASE INHIBITORS		
(57) Abstract A new use for HMG-CoA reductase inhibitors is provided. In the instant invention, HMG-CoA reductase inhibitors are found to upregulate endothelial cell Nitric Oxide Synthase activity through a mechanism other than preventing the formation of oxidative-LDL. As a result, HMG-CoA reductase inhibitors are useful in treating or preventing conditions that result from the abnormally low expression and/or activity of endothelial cell Nitric Oxide Synthase. Such conditions include pulmonary hypertension, ischemic stroke, impotence, heart failure, hypoxia-induced conditions, insulin deficiency, progressive renal disease, gastric or esophageal motility syndrome, etc. Subjects thought to benefit mostly from such treatments include nonhyperlipidemics and nonhypercholesterolemics, but not necessarily exclude hyperlipidemics and hypercholesterolemics.		

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**UPREGULATION OF TYPE III ENDOTHELIAL CELL NITRIC OXIDE
SYNTHASE BY HMG-CoA REDUCTASE INHIBITORS**

Field of the Invention

5 This invention describes the new use of HMG-CoA reductase inhibitors as upregulators of Type III endothelial cell Nitric Oxide Synthase. Further, this invention describes methods that employ HMG-CoA reductase inhibitors to treat conditions that result from the abnormally low expression and/or activity of endothelial cell Nitric Oxide Synthase in a subject.

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Background of the Invention

Nitric oxide (NO) has been recognized as an unusual messenger molecule with many physiologic roles, in the cardiovascular, neurologic and immune systems (Griffith, TM et al., *J Am Coll Cardiol*, 1988, 12:797-806). It mediates blood vessel relaxation, neurotransmission
15 and pathogen suppression. NO is produced from the guanidino nitrogen of L-arginine by NO Synthase (Moncada, S and Higgs, EA, *Eur J Clin Invest*, 1991, 21(4):361-374). In mammals, at least three isoenzymes of NO Synthase have been identified. Two, expressed in neurons (nNOS) and endothelial cells (Type III-ecNOS), are calcium-dependent, whereas the third is calcium-independent and is expressed by macrophages and other cells after induction with
20 cytokines (Type II-iNOS) (Bredt, DS and Snyder, SH, *Proc Natl Acad Sci USA*, 1990, 87:682-685, Janssens, SP et al., *J Biol Chem*, 1992, 267:22964, Lyons, CR et al., *J Biol Chem*, 1992, 267:6370-6374). The various physiological and pathological effects of NO can be explained by its reactivity and different routes of formation and metabolism.

Recent studies suggest that a loss of endothelial-derived NO activity may contribute to
25 the atherogenic process (O'Driscoll, G, et al., *Circulation*, 1997, 95:1126-1131). For example, endothelial-derived NO inhibits several components of the atherogenic process including monocyte adhesion to the endothelial surface (Tsao, PS et al., *Circulation*, 1994, 89:2176-2182), platelet aggregation (Radomski, MW, et al., *Proc Natl Acad Sci USA*, 1990, 87:5193-5197), vascular smooth muscle cell proliferation (Garg, UC and Hassid, A, *J Clin Invest*, 1989, 83:1774-1777), and vasoconstriction (Tanner, FC et al., *Circulation*, 1991, 83:2012-2020). In addition, NO can prevent oxidative modification of low-density
30 lipoprotein (LDL) which is a major contributor to atherosclerosis, particularly in its oxidized

form (Cox, DA and Cohen, ML, *Pharm Rev*, 1996, 48:3-19).

It has been shown in the prior art that hypoxia downregulates ecNOS expression and/or activity via decreases in both ecNOS gene transcription and mRNA stability (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666, Shaul, PW et al., *Am J Physiol*, 1997, 272:

5 L1005-L1012). Thus, ischemia-induced hypoxia may produce deleterious effects, in part, through decreases in ecNOS activity.

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase is the microsomal enzyme that catalyzes the rate limiting reaction in cholesterol biosynthesis (HMG-CoA6Mevalonate). An HMG-CoA reductase inhibitor inhibits HMG-CoA reductase, and as a
10 result inhibits the synthesis of cholesterol. A number of HMG-CoA reductase inhibitors has been used to treat individuals with hypercholesterolemia. Clinical trials with such compounds have shown great reductions of cholesterol levels in hypercholesterolemic patients. Moreover, it has been shown that a reduction in serum cholesterol levels is correlated with improved endothelium-dependent relaxations in atherosclerotic vessels (Treasure, CB et al., *N Engl J*
15 *Med*, 1995, 332:481-487). Indeed, one of the earliest recognizable benefits after treatment with HMG-CoA reductase inhibitors is the restoration of endothelium-dependent relaxations or ecNOS activity (*supra*, Anderson, TJ et al., *N Engl J Med*, 1995, 332:488-493).

Although the mechanism by which HMG-CoA reductase inhibitors restore endothelial function is primarily attributed to the inhibition of hepatic HMG-CoA reductase and the
20 subsequent lowering of serum cholesterol levels, little is known on whether inhibition of endothelial HMG-CoA reductase has additional beneficial effects on endothelial function.

Pulmonary hypertension is a major cause of morbidity and mortality in individuals exposed to hypoxic conditions (Scherrer, U et al., *N Engl J Med*, 1996, 334:624-629). Recent studies demonstrate that pulmonary arterial vessels from patients with pulmonary
25 hypertension have impaired release of NO (Giaid, A and Saleh, D, *N Engl J Med*, 1995, 333:214-221, Shaul, PW, *Am J Physiol*, 1997, 272: L1005-L1012). Additionally, individuals with pulmonary hypertension demonstrate reduced levels of ecNOS expression in their pulmonary vessels and benefit clinically from inhalation nitric oxide therapy (Roberts, JD et al., *N Engl J Med*, 1997, 336:605-610, Kouyoumdjian, C et al., *J Clin Invest*, 1994,
30 94:578-584). Conversely, mutant mice lacking ecNOS gene or newborn lambs treated with the ecNOS inhibitor, Nw-monomethyl-L-arginine (LNMA), develop progressive elevation of pulmonary arterial pressures and resistance (Steudel, W et al., *Circ Res*, 1997, 81:34-41,

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Fineman, JR et al., *J Clin Invest*, 1994, 93:2675-2683). It has also been shown in the prior art that hypoxia causes pulmonary vasoconstriction via inhibition of endothelial cell nitric oxide synthase (ecNOS) expression and activity (Adnot, S et al., *J Clin Invest*, 1991, 87:155-162, Liao, JK et al., *J Clin Invest*, 1995, 96, 2661-2666). Hence, hypoxia-mediated
5 downregulation of ecNOS may lead to the vasoconstrictive and structural changes associated with pulmonary hypertension.

Often cited as the third most frequent cause of death in the developed countries, stroke has been defined as the abrupt impairment of brain function caused by a variety of pathologic changes involving one or several intracranial or extracranial blood vessels.
10 Approximately 80% of all strokes are ischemic strokes, resulting from restricted blood flow. Mutant mice lacking the gene for ecNOS are hypertensive (Huang, PL et al., *Nature*, 1995, 377:239-242, Steudel, W et al., *Circ Res*, 1997, 81:34-41) and develop greater intimal smooth muscle proliferation in response to cuff injury. Furthermore, occlusion of the middle cerebral artery results in 21% greater infarct size in "ecNOS knockout" mice compared to wildtype
15 mice (Huang, Z et al., *J Cereb Blood Flow Metab*, 1996, 16:981-987). These findings suggest that the ecNOS production may play a role in cerebral infarct formation and sizes. Additionally, since most patients with ischemic strokes have average or normal cholesterol levels, little is known on what the potential benefits of HMG-CoA reductase inhibitor administration would be in cerebrovascular events.

20 There exists a need to identify agents that improve endothelial cell function.

There also exists a need to identify agents that can be used acutely or in a prophylactic manner to treat conditions that result from low levels of endothelial cell Nitric Oxide Synthase.

25 Summary of the Invention

The invention involves the discovery that HMG-CoA reductase inhibitors can upregulate endothelial cell Nitric Oxide Synthase (Type III) activity other than through preventing the formation of oxidative LDL. It previously was believed that such reductase inhibitors functioned by lowering serum cholesterol levels by blocking hepatic conversion of
30 HMG-CoA to L-mevalonate in the cholesterol biosynthetic pathway. It has been discovered, surprisingly, that HMG-CoA reductase inhibitors can increase Nitric Oxide Synthase activity by effects directly on endothelial rather than hepatic HMG-CoA reductase. This upregulation

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of activity does not depend upon a decrease in cholesterol synthesis and in particular does not depend upon a decrease in the formation of ox-LDL. The invention, therefore, is useful whenever it is desirable to restore endothelial cell Nitric Oxide Synthase activity or increase such activity in an affected cell or tissue. The tissue is defined as to include both the cells in
5 the vasculature supplying nutrients to the tissue, as well as cells of the tissue that express endothelial cell Nitric Oxide Synthase.

Nitric Oxide Synthase activity is involved in many conditions, including impotence, heart failure, gastric and esophageal motility disorders, kidney disorders such as kidney hypertension and progressive renal disease, insulin deficiency, etc. Individuals with such
10 conditions would benefit from increased endothelial cell Nitric Oxide Synthase activity. It also was known that individuals with pulmonary hypertension demonstrate reduced levels of Nitric Oxide Synthase expression in their pulmonary vessels and benefit clinically from inhalation of Nitric Oxide. The invention therefore is particularly useful for treating pulmonary hypertension. It also has been demonstrated that hypoxia causes an inhibition of
15 endothelial cell Nitric Oxide Synthase activity. The invention therefore is useful for treating subjects with hypoxia-induced conditions. It also has been discovered, surprisingly, that HMG-CoA reductase inhibitors are useful for reducing brain injury that occurs following a stroke.

According to one aspect of the invention, a method is provided for increasing
20 endothelial cell Nitric Oxide Synthase activity in a nonhypercholesterolemic subject who would benefit from increased endothelial cell Nitric Oxide Synthase activity in a tissue. The method involves administering to a nonhypercholesterolemic subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the tissue of the subject. The subject may also be
25 nonhyperlipidemic. In certain embodiments, the HMG-CoA reductase inhibitor is administered in an amount which alters the blood LDL cholesterol levels in the subject by less than 10%. The alteration may even be less than 5%. In certain embodiments the amount is sufficient to increase endothelial cell Nitric Oxide Synthase activity above normal baseline levels established by age-controlled groups, described in greater detail below.

30 The subject can have a condition characterized by an abnormally low level of endothelial cell Nitric Oxide Synthase activity which is hypoxia-induced. In other embodiments the subject can have a condition comprising an abnormally low level of

endothelial cell Nitric Oxide Synthase activity which is chemically induced. In still other embodiments the subject can have a condition comprising an abnormally low level of endothelial cell Nitric Oxide Synthase activity which is cytokine induced. In certain important embodiments, the subject has pulmonary hypertension or an abnormally elevated risk of pulmonary hypertension. In other important embodiments, the subject has experienced an ischemic stroke or has an abnormally elevated risk of an ischemic stroke. In still other important embodiments, the subject has heart failure or progressive renal disease. In yet other important embodiments, the subject is chronically exposed to hypoxic conditions.

According to any of the foregoing embodiments, the preferred HMG-CoA reductase inhibitor is selected from the group consisting of simvastatin and lovastatin. Likewise, in any of the foregoing embodiments, the method can further comprise co-administering an endothelial cell Nitric Oxide Synthase substrate and/or co-administering a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity. A preferred such agent is selected from the group consisting of estrogens and angiotensin-converting enzyme (ACE) inhibitors. The agents may be administered to a subject who has a condition or prophylactically to a subject who has a risk, and more preferably, an abnormally elevated risk, of developing a condition. The inhibitors also may be administered acutely.

According to another aspect of the invention, a method is provided for increasing endothelial cell Nitric Oxide Synthase activity in a subject to treat a nonhyperlipidemic condition favorably affected by an increase in endothelial cell Nitric Oxide Synthase activity in a tissue. Such conditions are exemplified above. The method involves administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the tissue of the subject. Important conditions are as described above. Also as described above, the method can involve co-administration of substrates of endothelial cell Nitric Oxide Synthase and/or a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity. Preferred compounds are as described above. As above, the reductase inhibitor can be administered, *inter alia*, acutely or prophylactically.

According to another aspect of the invention, a method is provided for reducing brain injury resulting from stroke. The method involves administering to a subject having an abnormally high risk of an ischemic stroke a HMG-CoA reductase inhibitor in an amount

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effective to increase endothelial cell Nitric Oxide Synthase activity in the brain of the subject. As above, important embodiments include the inhibitor being selected from the group consisting of simvastatin and lovastatin. Also as above, important embodiments include co-administering a substrate of endothelial cell Nitric Oxide Synthase and/or a nonHMG-CoA
5 reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity. Likewise, important embodiments include prophylactic and acute administration of the inhibitor.

According to another aspect of the invention, a method is provided for treating pulmonary hypertension. The method involves administering to a subject in need of such
10 treatment a HMG-CoA reductase inhibitor in an amount effective to increase pulmonary endothelial cell Nitric Oxide Synthase activity in the subject. Particularly important embodiments are as described above in connection with the methods for treating brain injury. Another important embodiment is administering the inhibitor prophylactically to a subject who has an abnormally elevated risk of developing pulmonary hypertension, including
15 subjects that are chronically exposed to hypoxic conditions.

According to another aspect of the invention, a method for treating heart failure is provided. The method involves administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase vascular endothelial cell Nitric Oxide Synthase activity in the subject. As discussed above, important embodiments
20 include prophylactic and acute administration of the inhibitor. Another important embodiment is treating a subject that is nonhyperlipidemic. Preferred compounds and co-administration schemes are as described above.

According to yet another aspect of the invention, a method is provided for treating progressive renal disease. The method involves administering to a subject in need of such
25 treatment a HMG-CoA reductase inhibitor in an amount effective to increase renal endothelial cell Nitric Oxide Synthase activity in the kidney of the subject. Important embodiments and preferred compounds and schemes of co-administration are as described above in connection with heart failure.

According to another aspect of the invention, a method for increasing blood flow in a
30 tissue of a subject is provided. The method involves administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the tissue of the subject. As discussed above, important

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embodiments include prophylactic and acute administration of the inhibitor. Preferred compounds and co-administration schemes are also as described above. Other important embodiments include co-administering a second agent to the subject with a condition treatable by the second agent in an amount effective to treat the condition, whereby the delivery of the
5 second agent to a tissue of the subject is enhanced as a result of the increased blood flow.

The invention also involves the use of HMG-CoA reductase inhibitors in the manufacture of medicaments for treating the above-noted conditions. Important conditions, compounds, etc. are as described above. The invention further involves pharmaceutical preparations that are cocktails of the HMG-CoA reductase inhibitors together with a
10 nonHMG-CoA reductase inhibitor agent that increases eNOS activity in a cell.

These and other aspects of the invention are described in greater detail below.

Brief Description of the Drawings

Figure 1. Western blots showing the effects of oxidized (ox)-LDL on eNOS protein
15 levels in the presence and absence of simvastatin. Figure 1A depicts the effects of increasing concentrations of simvastatin on eNOS protein levels. Figure 1B depicts the effects of increasing concentrations of simvastatin on eNOS protein levels in a time-dependent manner.

Figure 2. Northern blots showing the effects of ox-LDL on eNOS mRNA levels in the presence and absence of HMG-CoA reductase inhibitors. Figure 2A depicts the effects of
20 simvastatin on eNOS mRNA levels. Figure 2B depicts the effects of lovastatin on eNOS mRNA levels.

Figure 3. Western blots showing the concentration-dependent effects of simvastatin (Figure 3A) and lovastatin (Figure 3B) on eNOS protein levels after 48 hours.

Figure 4. Volume of cerebral infarction after 2 h filamentous middle cerebral artery
25 occlusion and 22 h reperfusion as % of control, with and without simvastatin treatment. Figure 4A depicts the cerebral infarction in wild-type SV-129 mice. Figure 4B depicts the cerebral infarction in eNOS-deficient mice.

Detailed Description of the Invention

30 The invention is useful whenever it is desirable to increase endothelial cell Nitric Oxide Synthase (Type III isoform) activity in a subject. Nitric Oxide Synthase is the enzyme that catalyzes the reaction that produces nitric oxide from the substrate L-arginine. As the

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name implies, endothelial cell nitric oxide Synthase refers to the Type III isoform of the enzyme found in the endothelium.

By "ecNOS activity", it is meant the ability of a cell to generate nitric oxide from the substrate L-arginine. Increased ecNOS activity can be accomplished in a number of different ways. For example, an increase in the amount of ecNOS protein or an increase in the activity of the protein (while maintaining a constant level of the protein) can result in increased "activity". An increase in the amount of protein available can result from increased transcription of the ecNOS gene, increased stability of the ecNOS mRNA or a decrease in ecNOS protein degradation.

10 The ecNOS activity in a cell or in a tissue can be measured in a variety of different ways. A direct measure would be to measure the amount of ecNOS present. Another direct measure would be to measure the amount of conversion of arginine to citrulline by ecNOS or the amount of generation of nitric oxide by ecNOS under particular conditions, such as the physiologic conditions of the tissue. The ecNOS activity also can be measured more
15 indirectly, for example by measuring mRNA half-life (an upstream indicator) or by a phenotypic response to the presence of nitric oxide (a downstream indicator). One phenotypic measurement employed in the art is detecting endothelial dependent relaxation in response to a acetylcholine, which response is affected by ecNOS activity. The level of nitric oxide present in a sample can be measured using a nitric oxide meter. All of the foregoing
20 techniques are well known to those of ordinary skill in the art, and some are described in the examples below.

The present invention, by causing an increase in ecNOS activity, permits not only the re-establishment of normal base-line levels of ecNOS activity, but also allows increasing such activity above normal base-line levels. Normal base-line levels are the amounts of activity in
25 a normal control group, controlled for age and having no symptoms which would indicate alteration of endothelial cell Nitric Oxide Synthase activity (such as hypoxic conditions, hyperlipidemia and the like). The actual level then will depend upon the particular age group selected and the particular measure employed to assay activity. Specific examples of various measures are provided below. In abnormal circumstances, e.g. hypoxic conditions,
30 pulmonary hypertension, etc., endothelial cell Nitric Oxide Synthase activity is depressed below normal levels. Surprisingly, when using the reductase inhibitors according to the invention, not only can normal base-line levels be restored in such abnormal conditions, but

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endothelial cell Nitric Oxide Synthase activity can be increased desirably far above normal base-line levels of endothelial cell Nitric Oxide Synthase activity. Thus, "increasing activity" means any increase in endothelial cell Nitric Oxide Synthase activity in the subject resulting from the treatment with reductase inhibitors according to the invention, including, but not
5 limited to, such activity as would be sufficient to restore normal base-line levels and such activity as would be sufficient to elevate the activity above normal base-line levels.

As mentioned above, Nitric Oxide Synthase activity is involved in many conditions, including stroke, pulmonary hypertension, impotence, heart failure, gastric and esophageal motility disorders, kidney disorders such as kidney hypertension and progressive renal
10 disease, insulin deficiency, hypoxia-induced conditions, etc.' In one embodiment of the invention the decrease in endothelial cell Nitric Oxide Synthase activity is cytokine induced. Cytokines are soluble polypeptides produced by a wide variety of cells that control gene activation and cell surface molecule expression. They play an essential role in the development of the immune system and thus in the development of an immune response.
15 However, besides their numerous beneficial properties, they have also been implicated in the mechanisms for the development of a variety of inflammatory diseases. For example, the cytokines TNF-a and IL-1 are thought to be part of the disease causing mechanism of non-cholesterol induced atherosclerosis, transplant arterial sclerosis, rheumatoid arthritis, lupus, scleroderma, emphysema, etc. Subjects of such disorders exhibit lower levels of
20 endothelial cell Nitric Oxide Synthase activity (which is thus "cytokine induced"), and may benefit from therapy using the agents of the instant invention.

One important embodiment of the invention is treatment of ischemic stroke. Ischemic stroke (ischemic cerebral infarction) is an acute neurologic injury that results from a decrease in the blood flow involving the blood vessels of the brain. Ischemic stroke is divided into two
25 broad categories, thrombotic and embolic.

A surprising finding was made in connection with the treatment of ischemic stroke. In particular, it was discovered that treatment according to the invention can reduce the brain injury that follows an ischemic stroke. Brain injury reduction, as demonstrated in the examples below, can be measured by determining a reduction in infarct size in the treated
30 versus the control groups. Likewise, functional tests measuring neurological deficits provided further evidence of reduction in brain injury in the treated animals versus the controls. Cerebral blood flow also was better in the treated animals versus the controls. Thus, in the

various accepted models of brain injury following stroke, a positive effect was observed in the treated animals versus the control animals. It is believed that all of the foregoing positive results are attributable to the upregulation of endothelial cell Nitric Oxide Synthase activity, which is believed demonstrated in the examples below.

5 An important embodiment of the invention is treatment of a subject with an abnormally elevated risk of an ischemic stroke. As used herein, subjects having an abnormally elevated risk of an ischemic stroke are a category determined according to conventional medical practice. Typically, the risk factors associated with cardiac disease are the same as are associated with stroke. The primary risk factors include hypertension,
10 hypercholesterolemia, and smoking. In addition, atrial fibrillation or recent myocardial infarction are important risk factors.

 The treatment of stroke can be for patients who have experienced a stroke or can be a prophylactic treatment. If prophylactic, then the treatment is for subjects having an abnormally elevated risk of an ischemic stroke, as described above. If the subject has
15 experienced a stroke, then the treatment can include acute treatment. Acute treatment means administration of the HMG-CoA reductase inhibitors at the onset of symptoms of the condition or at the onset of a substantial change in the symptoms of an existing condition.

 Another important embodiment of the invention is treatment of pulmonary hypertension. Pulmonary hypertension is a disease characterized by increased pulmonary
20 arterial pressure and pulmonary vascular resistance. Hypoxemia, hypocapnia, and an abnormal diffusing capacity for carbon monoxide are almost invariable findings of the disease. Additionally, according to the present invention, patients with pulmonary hypertension also have reduced levels of ecNOS expression in their pulmonary vessels. Traditionally, the criteria for subjects with, or at risk for pulmonary hypertension are defined
25 on the basis of clinical and histological characteristics according to Heath and Edwards (*Circulation*, 1958, 18:533-547).

 Subjects may be treated prophylactically to reduce the risk of pulmonary hypertension or subjects with pulmonary hypertension may be treated long term and/or acutely. If the treatment is prophylactic, then the subjects treated are those with an abnormally elevated risk
30 of pulmonary hypertension. A subject with an abnormally elevated risk of pulmonary hypertension is a subject with chronic exposure to hypoxic conditions, a subject with sustained vasoconstriction, a subject with multiple pulmonary emboli, a subject with

cardiomegaly and/or a subject with a family history of pulmonary hypertension.

Another important embodiment of the invention involves treating hypoxia-induced conditions. Hypoxia as used herein is defined as the decrease below normal levels of oxygen in a tissue. Hypoxia can result from a variety of circumstances, but most frequently results from impaired lung function. Impaired lung function can be caused by emphysema, cigarette smoking, chronic bronchitis, asthma, infectious agents, pneumonitis (infectious or chemical), lupus, rheumatoid arthritis, inherited disorders such as cystic fibrosis, obesity, α_1 -antitrypsin deficiency and the like. It also can result from non-lung impairments such as from living at very high altitudes. Hypoxia can result in pulmonary vasoconstriction via inhibition of eNOS activity.

Another important embodiment of the invention is the treatment of heart failure. Heart failure is a clinical syndrome of diverse etiologies linked by the common denominator of impaired heart pumping and is characterized by the failure of the heart to pump blood commensurate with the requirements of the metabolizing tissues, or to do so only from an elevating filling pressure.

The invention involves treatment of the foregoing conditions using HMG-CoA reductase inhibitors. "HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A)" is the microsomal enzyme that catalyzes the rate limiting reaction in cholesterol biosynthesis (HMG-CoA6Mevalonate). An "HMG-CoA reductase inhibitor" inhibits HMG-CoA reductase, and therefore inhibits the synthesis of cholesterol. There is a large number of compounds described in the art that have been obtained naturally or synthetically, which have been seen to inhibit HMG-CoA reductase, and which form the category of agents useful for practicing the present invention. Traditionally these agents have been used to treat individuals with hypercholesterolemia. Examples include some which are commercially available, such as simvastatin (U.S. Patent No. 4, 444,784), lovastatin (U.S. Patent No. 4,231,938), pravastatin sodium (U.S. Patent No. 4,346,227), fluvastatin (U.S. Patent No. 4,739,073), atorvastatin (U.S. Patent No. 5,273,995), cerivastatin, and numerous others described in U.S. Patent No. 5,622,985, U.S. Patent No. 5,135,935, U.S. Patent No. 5,356,896, U.S. Patent No. 4,920,109, U.S. Patent No. 5,286,895, U.S. Patent No. 5,262,435, U.S. Patent No. 5,260,332, U.S. Patent No. 5,317,031, U.S. Patent No. 5,283,256, U.S. Patent No. 5,256,689, U.S. Patent No. 5,182,298, U.S. Patent No. 5,369,125, U.S. Patent No. 5,302,604, U.S. Patent No. 5,166,171, U.S. Patent No. 5,202,327, U.S. Patent No. 5,276,021, U.S. Patent No. 5,196,440,

U.S. Patent No. 5,091,386, U.S. Patent No. 5,091,378, U.S. Patent No. 4,904,646, U.S. Patent No. 5,385,932, U.S. Patent No. 5,250,435, U.S. Patent No. 5,132,312, U.S. Patent No. 5,130,306, U.S. Patent No. 5,116,870, U.S. Patent No. 5,112,857, U.S. Patent No. 5,102,911, U.S. Patent No. 5,098,931, U.S. Patent No. 5,081,136, U.S. Patent No. 5,025,000, U.S. Patent
5 No. 5,021,453, U.S. Patent No. 5,017,716, U.S. Patent No. 5,001,144, U.S. Patent No. 5,001,128, U.S. Patent No. 4,997,837, U.S. Patent No. 4,996,234, U.S. Patent No. 4,994,494, U.S. Patent No. 4,992,429, U.S. Patent No. 4,970,231, U.S. Patent No. 4,968,693, U.S. Patent No. 4,963,538, U.S. Patent No. 4,957,940, U.S. Patent No. 4,950,675, U.S. Patent No. 4,946,864, U.S. Patent No. 4,946,860, U.S. Patent No. 4,940,800, U.S. Patent No. 4,940,727,
10 U.S. Patent No. 4,939,143, U.S. Patent No. 4,929,620, U.S. Patent No. 4,923,861, U.S. Patent No. 4,906,657, U.S. Patent No. 4,906,624 and U.S. Patent No. 4,897,402. the disclosures of which patents are incorporated herein by reference.

Important embodiments of the invention involve populations never before treated with an HMG-CoA reductase inhibitor. Thus, the invention involves in certain aspects treatments
15 of individuals who are otherwise free of symptoms calling for treatment with an HMG-CoA reductase inhibitor. It is believed that the only clinically accepted such condition is hypercholesterolemia, wherein the reductase inhibitor is administered for the purpose of preventing the biosynthesis of cholesterol. Thus, in certain embodiments the treated population is nonhypercholesterolemic. In other embodiments, the subject is
20 nonhypertriglyceridemic. In still other embodiments, the subject is both nonhypercholesterolemic and nonhypertriglyceridemic, i.e., nonhyperlipidemic.

A nonhypercholesterolemic subject is one that does not fit the current criteria established for a hypercholesterolemic subject. A nonhypertriglyceridemic subject is one that does not fit the current criteria established for a hypertriglyceridemic subject (See, e.g.,
25 Harrison's Principles of Experimental Medicine, 13th Edition, McGraw-Hill, Inc., N.Y.). Hypercholesterolemic subjects and hypertriglyceridemic subjects are associated with increased incidence of premature coronary heart disease. A hypercholesterolemic subject has an LDL level of >160 mg/dL or >130 mg/dL and at least two risk factors selected from the group consisting of male gender, family history of premature coronary heart disease, cigarette
30 smoking (more than 10 per day), hypertension, low HDL (<35 mg/dL), diabetes mellitus, hyperinsulinemia, abdominal obesity, high lipoprotein (a), and personal history of cerebrovascular disease or occlusive peripheral vascular disease. A hypertriglyceridemic

subject has a triglyceride (TG) level of >250 mg/dL. Thus, a hyperlipidemic subject is defined as one whose cholesterol and triglyceride levels equal or exceed the limits set as described above for both the hypercholesterolemic and hypertriglyceridemic subjects.

The invention also involves the co-administration of agents that are not HMG-CoA reductase inhibitors but that can act cooperatively, additively or synergistically with such HMG-CoA reductase inhibitors to increase ecNOS activity. Thus, ecNOS substrates which are converted by ecNOS to nitric oxide can be co-administered with the HMG-CoA reductase inhibitors according to the invention. Such ecNOS substrates may be natural or synthetic, although the preferred substrate is L-arginine.

Likewise, there are other agents besides HMG-CoA reductase inhibitors that are not substrates of ecNOS and that can increase ecNOS activity. Examples of categories of such agents are estrogens and ACE inhibitors. Estrogens are a well defined category of molecules known by those of ordinary skill in the art, and will not be elaborated upon further herein. All share a high degree of structural similarity. ACE inhibitors also have been well characterized, although they do not always share structural homology.

Angiotensin converting enzyme, or ACE, is an enzyme which catalyzes the conversion of angiotensin I to angiotensin II. ACE inhibitors include amino acids and derivatives thereof, peptides, including di and tri peptides and antibodies to ACE which intervene in the renin-angiotensin system by inhibiting the activity of ACE thereby reducing or eliminating the formation of pressor substance angiotensin II. ACE inhibitors have been used medically to treat hypertension, congestive heart failure, myocardial infarction and renal disease. Classes of compounds known to be useful as ACE inhibitors include acylmercapto and mercaptoalkanoyl prolines such as captopril (US Patent Number 4,105,776) and zofenopril (US Patent Number 4,316,906), carboxyalkyl dipeptides such as enalapril (US Patent Number 4,374,829), lisinopril (US Patent Number 4,374,829), quinapril (US Patent Number 4,344,949), ramipril (US Patent Number 4,587,258), and perindopril (US Patent Number 4,508,729), carboxyalkyl dipeptide mimics such as cilazapril (US Patent Number 4,512,924) and benazapril (US Patent Number 4,410,520), phosphinylalkanoyl prolines such as fosinopril (US Patent Number 4,337,201) andtrandolopril.

Estrogens upregulate Nitric Oxide Synthase expression whereas ACE inhibitors do not affect expression, but instead influence the efficiency of the action of Nitric Oxide Synthase on L-arginine. Thus, activity can be increased in a variety of ways. In general, activity is

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increased by the reductase inhibitors of the invention by increasing the amount of the active enzyme present in a cell versus the amount present in a cell absent treatment with the reductase inhibitors according to the invention.

The reductase inhibitors are administered in effective amounts. In general, an
5 effective amount is any amount that can cause an increase in Nitric Oxide Synthase activity in a desired tissue, and preferably in an amount sufficient to cause a favorable phenotypic change in the condition such as a lessening, alleviation or elimination of a symptom or of a condition.

In general, an effective amount is that amount of a pharmaceutical preparation that
10 alone, or together with further doses, produces the desired response. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently or delaying the onset of or preventing the disease or condition from occurring. This can be monitored by routine methods. Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000 mg/kg per day.
15 It is expected that doses ranging from 50-500 mg/kg will be suitable, preferably orally and in one or several administrations per day.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the
20 specific route of administration and like factors within the knowledge and expertise of the health practitioner. Lower doses will result from certain forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses
25 per day are contemplated to achieve appropriate systemic levels of compounds. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

30 The reductase inhibitors useful according to the invention may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers,

diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of reductase inhibitors, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using

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suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the active compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di-and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the active compound is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,667,014, 4,748,034 and 5,239,660 and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,832,253, and 3,854,480. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be desirable. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

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According to another aspect of the invention, a method for increasing blood flow in a tissue of a subject is provided. The method involves administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the tissue of the subject.

5 In important embodiments a second agent is co-administered to a subject with a condition treatable by the second agent in an amount effective to treat the condition, whereby the delivery of the second agent to a tissue of the subject is enhanced as a result of the increased blood flow from administering the first agent of the invention (an agent that disrupts actin cytoskeletal organization). The "second agent" may be any pharmacological compound
10 or diagnostic agent, as desired.

Examples of categories of pharmaceutical agents include: adrenergic agent; adrenocortical steroid; adrenocortical suppressant; alcohol deterrent; aldosterone antagonist; amino acid; ammonia detoxicant; anabolic; analeptic; analgesic; androgen; anesthesia, adjunct to; anesthetic; anorectic; antagonist; anterior pituitary suppressant; anthelmintic;
15 anti-acne agent; anti-adrenergic; anti-allergic; anti-amebic; anti-androgen; anti-anemic; anti-anginal; anti-anxiety; anti-arthritic; anti-asthmatic; anti-atherosclerotic; antibacterial; anticholelithic; anticholelithogenic; anticholinergic; anticoagulant; anticoccidal; anticonvulsant; antidepressant; antidiabetic; antidiarrheal; antidiuretic; antidote; anti-emetic; anti-epileptic; anti-estrogen; antifibrinolytic; antifungal; antiglaucoma agent;
20 antihemophilic; antihemorrhagic; antihistamine; antihyperlipidemia; antihyperlipoproteinemic; antihypertensive; anti-infective; anti-infective, topical; anti-inflammatory; antikeratinizing agent; antimalarial; antimicrobial; antimigraine; antimitotic; antimycotic, antinauseant, antineoplastic, antineutropenic, antiobsessional agent; antiparasitic; antiparkinsonian; antiperistaltic, antipneumocystic; antiproliferative; antiprostatic
25 hypertrophy; antiprotozoal; antipruritic; antipsychotic; antirheumatic; antischistosomal; antiseborrheic; antisecretory; antispasmodic; antithrombotic; antitussive; anti-ulcerative; anti-urolithic; antiviral; appetite suppressant; benign prostatic hyperplasia therapy agent; blood glucose regulator; bone resorption inhibitor; bronchodilator; carbonic anhydrase inhibitor; cardiac depressant; cardioprotectant; cardiotonic; cardiovascular agent; choleric;
30 cholinergic; cholinergic agonist; cholinesterase deactivator; coccidiostat; cognition adjuvant; cognition enhancer; depressant; diagnostic aid; diuretic; dopaminergic agent; ectoparasiticide; emetic; enzyme inhibitor; estrogen; fibrinolytic; fluorescent agent; free oxygen radical

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scavenger; gastrointestinal motility effector; glucocorticoid; gonad-stimulating principle; hair growth stimulant; hemostatic; histamine H2 receptor antagonists; hormone; hypocholesterolemic; hypoglycemic; hypolipidemic; hypotensive; imaging agent; immunizing agent; immunomodulator; immunoregulator; immunostimulant; immunosuppressant;

5 impotence therapy adjunct; inhibitor; keratolytic; LNRH agonist; liver disorder treatment; luteolysin; memory adjuvant; mental performance enhancer; mood regulator; mucolytic; mucosal protective agent; mydriatic; nasal decongestant; neuromuscular blocking agent; neuroprotective; NMDA antagonist; non-hormonal sterol derivative; oxytocic; plasminogen activator; platelet activating factor antagonist; platelet aggregation inhibitor; post-stroke and

10 post-head trauma treatment; potentiator; progestin; prostaglandin; prostate growth inhibitor; prothyrotropin; psychotropic; pulmonary surface; radioactive agent; regulator; relaxant; repartitioning agent; scabicide; sclerosing agent; sedative; sedative-hypnotic; selective adenosine A1 antagonist; serotonin antagonist; serotonin inhibitor; serotonin receptor antagonist; steroid; stimulant; suppressant; symptomatic multiple sclerosis; synergist; thyroid

15 hormone; thyroid inhibitor; thyromimetic; tranquilizer; treatment of amyotrophic lateral sclerosis; treatment of cerebral ischemia; treatment of Paget's disease; treatment of unstable angina; uricosuric; vasoconstrictor; vasodilator; vulnerary; wound healing agent; xanthine oxidase inhibitor.

In another aspect of the invention, the reductase inhibitor is "co-administered," which

20 means administered substantially simultaneously with another agent. By substantially simultaneously, it is meant that the reductase inhibitor is administered to the subject close enough in time with the administration of the other agent (e.g., a nonHMG-CoA reductase inhibitor agent, a "second agent", etc.), whereby the two compounds may exert an additive or even synergistic effect, i.e. on increasing eNOS activity or on delivering a second agent to a

25 tissue via increased blood flow.

Examples

"Upregulation of endothelial cell Nitric Oxide Synthase by HMG CoA Reductase Inhibitors"

Experimental Procedures

30 All standard culture reagents were obtained from JRH Bioscience (Lenexa, KS). Unless indicated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). [a-³²P]CTP (3000 Ci/mmol) was supplied by New England Nuclear. Purified human

LDL was obtained from Calbiochem (San Diego, CA; lot#730793) and Biomedical Technologies Inc. (Stoughton, MA; lot#9030197). The level of endotoxin was determined by the chromogenic Limulus amebocyte assay (BioWhittaker Inc., Walkersville, MD). The antibody detection kit (Enhanced Chemiluminescence) and the nylon nucleic acid (Hybond) and protein (PVDF) transfer membranes were purchased from Amersham Corp. (Arlington Heights, IL). Simvastatin and lovastatin were obtained from Merck, Sharp, and Dohme, Inc. (West Point, PA). Since endothelial cells lack lactonases to process simvastatin and lovastatin to their active forms, these HMG-CoA reductase inhibitors were chemically activated prior to their use as previously described (Laufs, U et al., *J Biol Chem*, 1997, 272:31725-31729).

10

Cell Culture:

Human endothelial cells were harvested from saphenous veins and cultured as described (15). For transfection studies, bovine aortic endothelial cells of less than 3 passages were cultured in a growth medium containing DMEM (Dulbecco's Modified Eagle's Medium), 5 mmol/L L-glutamine (Gibco), and 10% fetal calf serum (Hyclone Lot#1114577). For all experiments, the endothelial cells were placed in 10% lipoprotein-deficient serum (Sigma, Lot#26H94031) for 48 h prior to treatment conditions. In the indicated experiments, endothelial cells were pretreated with actinomycin D (5 mg/ml) for 1 h prior to treatment with ox-LDL and/or simvastatin. Cellular viability as determined by cell count, morphology, and Trypan blue exclusion was maintained for all treatment conditions.

20

Preparation of LDL:

The LDL was prepared by discontinuous ultracentrifugation according to the method of Chung et al. with some modification (*Methods Enzymol*, 1984, 128:181-209). Fresh plasma from a single donor was anticoagulated with heparin and filtered through a Sephadex G-25 column equilibrated with PBS. The density was adjusted to 1.21 g/ml by addition of KBr (0.3265 g/ml plasma). A discontinuous NaCl/KBr gradient was established in Beckman Quick-Seal centrifuge tubes (5.0 ml capacity) by layering 1.5 ml of density-adjusted plasma under 3.5 ml of 0.154 M NaCl in Chelex-100-treated water (BioRad, Hercules, CA). After ultracentrifugation at 443,000 x g and 7°C for 45 min in a Beckman Near Vertical Tube 90 rotor (Beckman L8-80M ultracentrifuge), the yellow band in the upper middle of the tube corresponding to LDL was removed by puncturing with a needle and withdrawing into a

30

syringe. The KBr was removed from the LDL by dialyzing with three changes of sterile PBS, pH 7.4, containing 100 :g/ml polymyxin B.

The purity of the LDL samples was confirmed by SDS/polyacrylamide and cellulose acetate gel electrophoresis. Cholesterol and triglyceride content were determined as previously described (Liao, JK et al., *J Biol Chem*, 1995, 270:319-324.). The LDL protein concentration was determined by the method of Lowry et al., (*J Biol Chem*, 1951, 193:265-275.). For comparison, commercially-available LDL (Biomedical Technologies Inc., Stoughton, MA; Calbiochem, San Diego, CA) were characterized and used in selected experiments.

10

Oxidation of LDL:

Oxidized LDL was prepared by exposing freshly-isolated LDL to CuSO₄ (5-10 mM) at 37°C for various duration (6-24 h). The reaction was stopped by dialyzing with three changes of sterile buffer (150 :mol/L NaCl, 0.01% EDTA and 100 :g/ml polymyxin B, pH 7.4) at 4°C. The degree of LDL oxidation was estimated by measuring the amounts of thiobarbituric acid reactive substances (TBARS) produced using a fluorescent assay for malondialdehyde as previously described (Yagi, KA, *Biochem Med*, 1976, 15:212-21.). The extent of LDL modification was expressed as nanomoles of malondialdehyde per mg of LDL protein. Only mild to moderate ox-LDL with TBARS values between 12 and 16 nmol/mg LDL protein (i.e. 3 to 4 nmol/mg LDL cholesterol) were used in this study. All oxidatively-modified LDL samples were used within 24 h of preparation.

Northern Blotting:

Equal amounts of total RNA (10-20 mg) were separated by 1.2% formaldehyde-agarose gel electrophoresis and transferred overnight onto Hybond nylon membranes. Radiolabeling of human full-length ecNOS cDNA (Verbeuren, TJ et al., *Circ Res*, 1986, 58:552-564, Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666) was performed using random hexamer priming, [α -³²P]CTP, and Klenow (Pharmacia). The membranes were hybridized with the probes overnight at 45°C in a solution containing 50% formamide, 5 X SSC, 2.5 X Denhardt's Solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 mg/ml salmon sperm DNA. All Northern blots were subjected to stringent washing conditions (0.2 X SSC/0.1% SDS at 65°C) prior to autoradiography. RNA loading was

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determined by rehybridization with human GAPDH probe.

Western Blotting:

Cellular proteins were prepared and separated on SDS/PAGE as described (Liao, JK et al., *J Biol Chem*, 1995, 270:319-324.). Immunoblotting was performed using a murine
5 monoclonal antibody to human ecNOS (1:400 dilution, Transduction Laboratories, Lexington, KY). Immunodetection was accomplished using a sheep anti-mouse secondary antibody (1:4000 dilution) and the enhanced chemiluminescence (ECL) kit (Amersham Corp., Arlington Heights, IL). Autoradiography was performed at 23°C and the appropriate
10 exposures were quantitated by densitometry.

Assay for ecNOS Activity:

The ecNOS activity was determined by a modified nitrite assay as previously described (Misko, TP et al., *Analytical Biochemistry*, 1993, 214:11-16. Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). Briefly, endothelial cells were treated for 24 h with ox-LDL in
15 the presence and absence of simvastatin (0.1 to 1 mM). After treatment, the medium was removed, and the cells were washed and incubated for 24 h in phenol red-free medium. After 24 h, 300 :l of conditioned medium was mixed with 30 :l of freshly prepared 2,3-diaminonaphthalene (1.5 mmol/L DAN in 1 mol/L HCl). The mixture was protected from
20 light and incubated at 20°C for 10 min. The reaction was terminated with 15 :l of 2.8 mol/L NaOH. Fluorescence of 1-(H)-naphthotriazole was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. Standard curves were constructed with known amounts of sodium nitrite. Nonspecific fluorescence was determined in the presence of LNMA (5 mmol/L).

25

Nuclear Run-on Assay:

Confluent endothelial cells (~5 x 10⁷ cells) grown in LPDS were treated with simvastatin (1 mM) or 95%O₂ for 24 h. Nuclei were isolated and in vitro transcription was performed as previously described (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). Equal
30 amounts (1 mg) of purified, denatured full-length human ecNOS, human β -tubulin (ATCC #37855), and linearized pGEM-3z cDNA were vacuum-transferred onto nitrocellulose membranes using a slot blot apparatus (Schleicher & Schuell). Hybridization of radiolabeled

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mRNA transcripts to the nitrocellulose membranes was carried out at 45°C for 48 h in a buffer containing 50% formamide, 5 X SSC, 2.5 X Denhardt's solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 mg/ml salmon sperm DNA. The membranes were then washed with 1 x SSC/0.1% SDS for 1 h at 65°C prior to autoradiography for 72 h at -80°C.

5

Transfection Assays:

For transient transfections, bovine rather than human endothelial cells were used because of their higher transfection efficiency by the calcium-phosphate precipitation method (12% vs <4%) (Graham, FL and Van der Erb, AJ, *Virology*, 1973, 52:456-457). We used the human ecNOS promoter construct, F1.LUC, which contains a -1.6 kb 5'-upstream sequence linked to the luciferase reporter gene as described by Zhang et al. (*J Biol Chem*, 1995, 270:15320-15326). Bovine endothelial cells (60%-70% confluent) were transfected with 30 mg of the indicated constructs: p.LUC (no promoter), pSV2.LUC (SV40 early promoter), or F1.LUC. As an internal control for transfection efficiency, pCMV.bGal plasmid (10 mg) was co-transfected in all experiments. Preliminary results using b-galactosidase staining indicate that cellular transfection efficiency was approximately 10% to 14%.

Endothelial cells were placed in lipoprotein-deficient serum for 48 h after transfection and treated with ox-LDL (50 mg/ml, TBARS 12.4 nmol/mg) in the presence and absence of simvastatin (1 mM) for an additional 24 h. The luciferase and b-galactosidase activities were determined by a chemiluminescence assay (Dual-Light, Tropix, Bedford, MA) using a Berthold L9501 luminometer. The relative promoter activity was calculated as the ratio of luciferase-to β -galactosidase activity. Each experiment was performed three times in triplicate.

25 Data Analysis:

Band intensities were analyzed densitometrically by the National Institutes of Health Image program (Rasband, W, NIH Image program, v 1.49, National Institutes of Health, Bethesda, 1993). All values are expressed as mean \pm SEM compared to controls and among separate experiments. Paired and unpaired Student's t tests were employed to determine any significant changes in densitometric values, nitrite production, and promoter activities. A significant difference was taken for P values less than 0.05.

30

Example 1: Cell Culture

Relatively pure (>95%) human endothelial cell cultures were confirmed by their morphological features (i.e. cuboidal, cobble-stone, contact inhibited) using phase-contrast microscopy and by immunofluorescent staining with anti-Factor VIII antibodies (Gerson, RJ et al., *Am J Med*, 1989, 87:28-38). For all experimental conditions, there were no observable adverse effects of ox-LDL or HMG-CoA reductase inhibitors on cellular morphology, cell number, immunofluorescent staining, and Trypan blue exclusion (>95%). Higher concentrations of ox-LDL (>100 mg/ml) with greater oxidative modification (i.e. TBARS values of >30 nmol/mg) caused vacuolization and some detachment of endothelial cells after 24 h. Neither simvastatin (0.01 to 0.1 mmol/L) nor lovastatin (10 mmol/L) produced any noticeable adverse effects on human endothelial cell for up to 96 h. However, higher concentrations of simvastatin (>15 mmol/L) or lovastatin (>50 mmol/L) caused cytotoxicity after 36 h, and therefore, were not used.

Example 2: Characterization of LDL

SDS/polyacrylamide gel electrophoresis of native or unmodified LDL revealed a single band (~510 kD) corresponding to ApoB-100 (data not shown). Similarly, cellulose acetate electrophoresis revealed only one band corresponding to the presence of a single class of low-density lipids (density of 1.02 to 1.06 g/ml). The LDL had a protein, cholesterol, and triglyceride concentration of 6.3 ± 0.2 , 2.5 ± 0.1 , and 0.5 ± 0.1 mg/ml, respectively. In contrast, lipoprotein-deficient serum was devoid of both apoB-100 protein and low-density lipid bands, and had non-detectable levels of cholesterol. There was no detectable level of endotoxin (<0.10 EU/ml) in the lipoprotein-deficient serum or ox-LDL samples by the chromogenic Limulus amebocyte assay.

In addition, there was no apparent difference between our own preparation and commercially-obtained LDL samples in terms of electrophoretic mobility. Native LDL had a TBARS value of 0.3 ± 0.2 nmol/mg, but after exposure to human saphenous vein endothelial cells in lipoprotein-deficient media for 72 h, this value increased to 3.1 ± 0.4 nmol/mg. Copper-oxidized LDL had TBARS values ranging from 4.6 ± 0.5 to 33.1 ± 5.2 nmol/mg. The degree of ox-LDL used in this study was mild to moderate with TBARS value ranging from 12 to 16 nmol/mg LDL protein (i.e. 3 to 4 nmol/mg LDL cholesterol).

Example 3: Effect of ox-LDL and HMG-CoA Reductase Inhibitors on ecNOS Protein

We have previously shown that ox-LDL (50 mg/ml) downregulates ecNOS expression (Liao, JK et al., *J Biol Chem*, 1995, 270:319-324). Compared to untreated cells, treatment with ox-LDL (50 mg/ml, TBARS 12.2 nmol/mg) caused a $54\% \pm 6\%$ decrease in ecNOS protein after 48 h ($p < 0.01$, $n=4$) (Western blots, 40 mg protein/lane, Figure 1A). There was no difference between our preparation of ox-LDL and commercially-available ox-LDL with similar TBARS values in terms of the degree of ecNOS downregulation. Addition of simvastatin (0.01 mmol/L) did not significantly affect the downregulation of ecNOS protein by ox-LDL ($57\% \pm 8\%$ decrease, $p > 0.05$, $n=4$). However, in the presence of 0.1 mmol/L of simvastatin, ox-LDL no longer produce any significant decrease in ecNOS protein levels ($4\% \pm 7\%$ decrease, $p < 0.01$, $n=4$). Higher concentrations of simvastatin (1 and 10 mmol/L) resulted in not only a reversal of ecNOS downregulation by ox-LDL, but also significant increases in ecNOS protein levels above baseline ($146\% \pm 9\%$ and $210\% \pm 12\%$, respectively, $p < 0.05$, $n=4$). Simvastatin or lovastatin (10 mmol/L) which were not chemically-activated had no effect on ecNOS expression.

In a time-dependent manner, treatment with ox-LDL (50 mg/ml, TBARS 12.2 nmol/mg) decreased ecNOS protein expression by 34% (5%, 67% (8% and 86 (5% after 24 h, 72 h, and 96 h, respectively ($p < 0.05$ for all values, $n=4$.) (Figure 1B). Compared to ox-LDL alone, co-treatment with simvastatin (0.1 mmol/L) attenuated the decrease in ecNOS protein level after 24 h (15% (2% vs 34% (5%, $p < 0.05$, $n=4$). Longer incubation with simvastatin (0.1 mmol/L) for 72 h and 96 h not only reversed ox-LDL's inhibitory effects on ecNOS expression, but also increased ecNOS protein levels by 110% (6% and 124% (6% above basal expression ($p < 0.05$, $n=4$). Thus, compared to ox-LDL alone, co-treatment with simvastatin produced a 1.3-, 3.3-and 8.9-fold increase ecNOS protein levels after 24 h, 72 h, and 96 h, respectively. These blots are representative of four separate experiments.

Example 4: Effect of ox-LDL and HMG-CoA Reductase Inhibitors on ecNOS mRNA

The effect of simvastatin on ecNOS mRNA levels occurred in a time-dependent manner and correlated with its effect on ecNOS protein levels. Northern blot analyses (Northern blots, 20 mg total RNA/lane, Figure 2A) showed that ox-LDL (50 mg/ml, TBARS 15.1 nmol/mg) produced a time-dependent $65 \pm 5\%$ and $91 \pm 4\%$ decrease in ecNOS mRNA levels after 48 h and 72 h, respectively ($p < 0.01$, $n=3$). Compared to ox-LDL at the indicated

time points, co-treatment with simvastatin 0.1 mmol/L) increased ecNOS mRNA levels by 6.3-fold after 48 h and 14.5-fold after 72 h ($p < 0.01$ for all values, $n=3$).

To determine whether treatment with another HMG-CoA reductase inhibitor have similar effect as simvastatin, we treated endothelial cells with lovastatin. Again, ox-LDL
5 decreased steady-state ecNOS mRNA by $52 \pm 5\%$ after 24 h ($p < 0.01$, $n=3$) (Figure 2B). Treatment with lovastatin (10 mmol/L) not only reversed the inhibitory effects of ox-LDL on ecNOS mRNA, but also caused a $40 \pm 9\%$ increase in ecNOS mRNA level compared to that of untreated cells. Compared to ox-LDL alone, co-treatment with lovastatin caused a 3.6-fold increase in ecNOS mRNA levels after 24 h. Treatment with lovastatin alone, however,
10 produced 36% increase in ecNOS mRNA levels compared to untreated cells ($p < 0.05$, $n=3$). Each experiment was performed three times with comparable results. The corresponding ethidium bromide-stained 28S band intensities were used to standardize loading conditions.

Example 5: Effect of ox-LDL and Simvastatin on ecNOS Activity

15 The activity of ecNOS was assessed by measuring the LNMA-inhibitable nitrite production from human endothelial cells (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). Basal ecNOS activity was 8.8 ± 1.4 nmol/500,000 cells/24 h. Treatment with ox-LDL (50 mg/ml, TBARS 16 nmol/mg) for 48 h decreased ecNOS-dependent nitrite production by $94 \pm 3\%$ (0.6 ± 0.5 nmol/500,000 cells/24 h, $p < 0.001$). Co-treatment with simvastatin (0.1
20 mmol/L) significantly attenuated this downregulation resulting in a $28 \pm 3\%$ decrease in ecNOS activity compared to untreated cells (6.4 ± 0.3 nmol/500,000 cells/24 h, $p < 0.05$). Co-treatment with a higher concentration of simvastatin (1 mmol/L) not only completely reversed the downregulation of ecNOS by ox-LDL, but also, resulted in a $45 \pm 6\%$ increase in ecNOS activity compared to baseline (12.8 ± 2.7 nmol/500,000 cells/24 h, $p < 0.05$).
25 Experiments were performed three times in duplicate.

Example 6: Effect of Simvastatin on ecNOS mRNA Stability

The post-transcriptional regulation of ecNOS mRNA was determined in the presence of the transcriptional inhibitor, actinomycin D (5 mg/ml). Oxidized LDL (50 mg/ml, TBARS
30 13.1 nmol/mg) shortened the half-life of ecNOS mRNA ($t_{1/2}$ 35 ± 3 h to 14 ± 2 h, $p < 0.05$, $n=3$). Co-treatment with simvastatin (0.1 mmol/L) prolonged the half-life of ecNOS mRNA by 1.6-fold ($t_{1/2}$ 22 ± 3 h, $p < 0.05$, $n=3$). Treatment with simvastatin alone prolonged

ecNOS mRNA half-life by 1.3-fold over baseline ($t_{1/2} 43 \pm 4$ h, $p < 0.05$, $n=3$). Band intensities of ecNOS mRNA (relative intensity) were plotted as a semi-log function of time (h). The data points obtained represented mean \pm SEM of three separate experiments.

5 Example 7: Effect of Simvastatin on ecNOS Gene Transcription

To determine whether the effects of simvastatin on ecNOS expression occurs at the level of ecNOS gene transcription, we performed nuclear run-on assays using endothelial cells treated with simvastatin (1 mmol/L) for 24 h. Preliminary studies using different amounts of radiolabelled RNA transcripts demonstrate that under our experimental conditions,
10 hybridization was linear and nonsaturable. The density of each ecNOS band was standardized to the density of its corresponding β -tubulin. The specificity of each band was determined by the lack of hybridization to the nonspecific pGEM cDNA vector. In untreated endothelial cells (control), there was constitutive ecNOS transcriptional activity (relative index of 1.0). Treatment with simvastatin (1 mmol/L) did not significantly affect ecNOS gene transcription
15 compared to that of untreated cells (relative index of 1.2 ± 0.3 , $p > 0.05$, $n=4$). However, treatment of endothelial cells with hyperoxia (95% O_2) significantly increased ecNOS gene expression (relative index of 2.5, $p < 0.05$, $n=4$). The blots shown are representative of four separate experiments.

To further confirm the effects of simvastatin on ecNOS gene transcription by a
20 different method, we transfected bovine aortic endothelial cells using a -1600 to +22 nucleotide ecNOS 5'-promoter construct linked to a luciferase reporter gene (F1.LUC) (Zhang, R et al., *J Biol Chem*, 1995, 270:15320-15326). This promoter construct contains putative cis-acting elements for activator protein (AP)-1 and -2, sterol regulatory element-1, retinoblastoma control element, shear stress response element (SSRE), nuclear factor-1
25 (NF-1), and cAMP response element (CRE). Treatment with ox-LDL (50 mg/ml, TBARS 14.5 nmol/mg), simvastatin (1 mol/L), alone or in combination, did not significantly affect basal F1 promoter activity. However, laminar fluid shear-stress (12 dynes/cm² for 24 h) was able to induce F1 promoter activity by 16-fold after 24 h (data not shown) indicating that the F1 promoter construct is functionally-responsive if presented with the appropriate stimulus.

30

Example 8: Effect of Simvastatin and Lovastatin on ecNOS Expression

To further characterize the effects of HMG-CoA reductase inhibitors on the

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upregulation ecNOS expression, we treated endothelial cells with simvastatin (0.1 mmol/L) for various durations (0-84 h). Treatment with simvastatin (0.1 mmol/L) increased ecNOS protein levels by 4 (6%, 21 (9%, 80 (8%, 90 (12%, and 95 (16% after 12 h, 24 h, 48 h, 72 h, and 84 h, respectively ($p < 0.05$ for all time points after 12 h, $n=4$). Higher concentrations of simvastatin similarly increased ecNOS protein levels, but in significantly less time compared to lower concentrations of simvastatin. (Western blots, 40 mg protein/lane).

In a concentration-dependent manner, treatment with simvastatin (0.01 to 10 mmol/L, 48 h) increased ecNOS expression by 1 (6%, 80 (8%, 190 (10% and 310 (20%, respectively ($p < 0.05$ for concentrations (0.1 mmol/L, $n=4$) (Figure 3A). The upregulation of ecNOS expression by simvastatin, therefore, is dependent upon both the concentration and duration of simvastatin treatment. For comparison, treatment with lovastatin (0.1 to 10 mmol/L, 48 h) also increased ecNOS expression in a concentration-dependant manner (10 (6%, 105 (8% and 180 (11%, respectively, $p < 0.05$ for concentrations > 0.1 mmol/L, $n=3$) (Figure 3B) but significantly less effectively than simvastatin at comparable concentrations. Therefore, at the same concentration, simvastatin had greater effects on ecNOS expression compared to lovastatin. These results are consistent with reported IC₅₀ values for simvastatin and lovastatin (4 nmol/L and 19 nmol/L, respectively) (Van Vliet, AK et al., *Biochem Pharmacol*, 1996, 52:1387-1392).

20 Example 9: Effect of L-Mevalonate on ecNOS Expression

To confirm that the effects of simvastatin on ecNOS expression were due to the inhibition of endothelial HMG CoA reductase, endothelial cells were treated with ox-LDL (50 mg/ml, TBARS 15.1 nmol/mg), simvastatin (1 mmol/L), alone or in combination, in the presence of L-mevalonate (100 mmol/L). Treatment with ox-LDL decreased ecNOS expression by 55% (6% after 48 h which was completely reversed and slightly upregulated in the presence of simvastatin (1 mmol/L) (150% (8% above basal expression) ($p < 0.05$ for both, $n=3$).

Compared to endothelial cells treated with ox-LDL and simvastatin, addition of L-mevalonate reduced ecNOS protein by $50\% \pm 5\%$ ($p < 0.05$, $n=3$). Furthermore, the upregulation of ecNOS expression by simvastatin alone (2.9-fold increase, $p < 0.05$, $n=3$) was completely reversed by co-treatment with L-mevalonate. Treatment with L-mevalonate alone did not have any appreciable effects on basal ecNOS expression ($p > 0.05$, $n=3$). Similar

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findings were also observed with L-mevalonate and lovastatin.

"HMG-CoA Reductase Inhibitors Reduce Cerebral Infarct Size by Upregulating endothelial cell Nitric Oxide Synthase"

5

Experimental Procedures

Cell Culture:

Human endothelial cells were harvested from saphenous veins using Type II collagenase (Worthington Biochemical Corp., Freehold, NJ) as previously described. Cells of
10 less than three passages were grown to confluence in a culture medium containing Medium 199, 20 mM HEPES, 50 mg/ml ECGS (Collaborative Research Inc., Bedford, MA), 100 mg/ml heparin sulfate, 5 mM L-glutamine (Gibco), 5% fetal calf serum (Hyclone, Logan, UT), and antibiotic mixture of penicillin (100 U/ml)/ streptomycin (100 mg/ml)/Fungizone (1.25 mg/ml). For all experiments, the endothelial cells were grown to confluence before any
15 treatment conditions. In some experiments, cells were pretreated with actinomycin D (5 mg/ml) for 1 h prior to treatment with HMG-CoA reductase inhibitors.

Exposure of Endothelial Cells to Hypoxia:

Confluent endothelial cells grown in 100 mm culture dishes were treated with
20 HMG-CoA reductase inhibitors and then placed without culture dish covers in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA). The chambers were gassed with 20% or 3% O₂, 5% CO₂, and balanced nitrogen for 10 min prior to sealing the chambers. The chambers were maintained in a 37°C incubator for various durations (0-48 h) and found to have less than 2% variation in O₂ concentration as previously described (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). Cellular confluence and viability were determined by cell
25 count, morphology, and trypan blue exclusion.

In vitro Transcription Assay:

Confluent endothelial cells (5 x 10⁷ cells) were treated with simvastatin (1 mM) in the
30 presence of 20% or 3% O₂ for 24 h. Nuclei were isolated and in vitro transcription was performed as previously described (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). Equal amounts (1 mg) of purified, denatured full-length human ecNOS, human β -tubulin (ATCC

#37855), and linearized pGEM-3z cDNA were vacuum-transferred onto nitrocellulose membranes using a slot blot apparatus (Schleicher & Schuell). Hybridization of radiolabeled mRNA transcripts to the nitrocellulose membranes was carried out at 45°C for 48 h in a buffer containing 50% formamide, 5 X SSC, 2.5 X Denhardt's solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 mg/ml salmon sperm DNA. The membranes were then washed with 1 x SSC/0.1% SDS for 1 h at 65°C prior to autoradiography for 72 h at -80°C. Band intensities were subjected to analyses by laser densitometry.

Assay for Nitrite Accumulation:

10 The amount of NO produced by ecNOS was determined by nitrite accumulation in the conditioned medium. Nitrite accumulation was determined by measuring the conversion of 2,3-diaminonaphthalene (1.5 mM of DAN in 1 M of HCl) and nitrite to 1-(H)-naphthotriazole as previously described (13,24). Nonspecific fluorescence was determined in the presence of LNMA (5 mM). Previous studies with nitrate reductase indicate that the nitrite to nitrate concentration in the medium was approximately 5:1 and that this ratio did not vary with exposure to 20% or 3% O₂ concentration.

Murine Model of Cerebral Vascular Ischemia:

Adult male (18-20 g) wildtype SV-129 mice (Taconic farm, Germantown, NY) and ecNOS mutant mice (Huang, PL et al., *Nature*, 1995, 377:239-242.) were subcutaneously-injected with 0.2, 2, or 20 mg of activated simvastatin per kg body weight or saline (control) once daily for 14 days. Ischemia was produced by occluding the left middle cerebral artery (MCA) with a coated 8.0 nylon monofilament under anesthesia as described (Huang, Z et al., *J Cereb Blood Flow Metab*, 1996, 16:981-987, Huang, Z et al., *Science*, 1994, 265:1883-1885, Hara, H et al., *J Cereb Blood Flow Metab*, 1997, 1:515-526). Arterial blood pressure, heart rate, arterial oxygen pressure, and partial pressure of carbon dioxide were monitored as described (Huang, Z et al., *J Cereb Blood Flow Metab*, 1996, 16:981-987, Huang, Z et al., *Science*, 1994, 265:1883-1885, Hara, H et al., *J Cereb Blood Flow Metab*, 1997, 1:515-526). The filaments were withdrawn after 2 hours and after 24 h, mice were either sacrificed or tested for neurological deficits using a well-established, standardized, observer-blinded protocol as described (Huang, Z et al., *J Cereb Blood Flow Metab*, 1996, 16:981-987, Huang, Z et al., *Science*, 1994, 265:1883-1885, Hara, H et al., *J Cereb Blood*

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Flow Metab, 1997, 1:515-526). The motor deficit score range from 0 (no deficit) to 2 (complete deficit).

Brains were divided into five coronal 2-mm sections using a mouse brain matrix (RBM-200C, Activated Systems, Ann Arbor, MI, USA). Infarction volume was quantitated with an image analysis system (M4, St. Catharines, Ontario, Canada) on 2% 2,3,5-triphenyltetrazolium chloride stained 2-mm slices. The levels of serum cholesterol, creatinine and transaminases were determined by the Tufts University Veterinary Diagnostic Laboratory (Grafton, MA).

10 Assay for ecNOS Activity from Tissues:

The ecNOS activities in mice aortae and brains were measured by the conversion of [³H]arginine to [³H]citrulline in the presence and absence of LNMA (5 mM) as described earlier.

15 Quantitative Reverse Transcription-Polymerase Chain Reaction:

Total RNA from mouse aortae and brains was isolated by the guanidinium isothiocyanate method and reverse transcribed using oligo-dT (mRNA Preamplification reagents; Gibco BRL) and Taq polymerase (Perkin-Elmer). One tenth of the sDNA was used as template for the PCR reaction. Approximately 0.2 nmol of the following primers amplifying a 254-bp fragment of murine ecNOS cDNA were used: 5'Primer: 5'-GGGCTCCCTCCTTCCGGCTGCCACC-3' (SEQ ID NO. 1) and 3'Primer: 5'-GGATCCCTGGAAAAGGCGGTGAGG-3' (SEQ ID NO. 2) (Hara, H et al., *J Cereb Blood Flow Metab*, 1997, 1:515-526). For amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 0.1 nmol of the following primers amplifying a 452-bp fragment were used: 5'Primer: 5'-ACCACAGTCCATGCCATCAC-3' (SEQ ID NO. 3) and 3' Primer: 5'-TCCACCACCCTGTTGCTGTA-3' (SEQ ID NO. 4). Denaturing was performed at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 60 s. Preliminary results indicated that the linear exponential phase for ecNOS and GAPDH polymerization was 30-35 cycles and 20-25 cycles, respectively.

30

Example 10: Cell Culture

Relatively pure (>98%) human saphenous vein endothelial cell cultures were

confirmed by their morphological features (ie. cuboidal, cobble-stone, contact inhibited) using phase-contrast microscopy and immunofluorescent-staining with antibodies to Factor VIII. There were no observable adverse effects of HMG-CoA reductase inhibitors, L-mevalonic acid, or hypoxia on cellular morphology. However, higher concentrations of simvastatin (>15 mmol/L) or lovastatin (>50 mmol/L) caused cytotoxicity after 36 h, and therefore, were not used. Otherwise, cellular confluency and viability as determined by trypan blue exclusion were maintained for all treatment conditions described.

Example 11: Effects of HMG-CoA Reductase Inhibitors on ecNOS Activity

10 The activity of ecNOS was assessed by measuring the LNMA-inhibitable nitrite accumulation from human endothelial cells (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). The ratio of nitrite to nitrate production under our culture condition was approximately 5:1 and was similar for hypoxia and normoxia. Basal ecNOS activity at 20% O₂ was 6.0 ± 3.3 nmol/500,000 cells/24 h. Exposure of endothelial cells to 3% O₂ for 24 h
15 decreased nitrite production by $75 \pm 14\%$ (1.5 ± 0.9 nmol/500,000 cells/24 h, $p < 0.01$). Treatment with simvastatin (1 mM) not only completely reversed the downregulation of ecNOS by hypoxia, but resulted in a 3-fold increase in ecNOS activity over basal activity (18 ± 5.0 nmol/500,000 cells/24 h, $p < 0.05$). This upregulation of ecNOS activity was attenuated by the addition of L-mevalonate (400 mM) (9.6 ± 1.3 nmol/500,000 cells/24 h, $p < 0.05$).
20 Interestingly, simvastatin (1 mM) alone upregulated nitrite production 5-fold (30 ± 6.5 nmol/500,000 cells/24 h, $p < 0.01$), which was completely blocked by L-mevalonate (400 mM) (8.6 ± 2.9 nmol/500,000 cells/24 h, $p < 0.05$). Similar findings were observed with lovastatin, but at 10-fold higher concentration compared to that of simvastatin.

25 Example 12: Effects of HMG-CoA Reductase Inhibitors on ecNOS Protein and mRNA Levels

In a concentration-dependent manner, treatment with simvastatin (0.01 to 10 mM, 48 h) increased ecNOS expression by 1 (6%, 80 (8%, 190 (10% and 310 (20%, respectively ($p < 0.05$ for concentrations (0.1 mM, $n=4$). Treatment with simvastatin (0.1 mM) increased ecNOS protein levels in a time-dependent manner by 4 (6%, 21 (9%, 80 (8%, 90 (12%, and
30 95 (16% after 12 h, 24 h, 48 h, 72 h, and 84 h, respectively ($p < 0.05$ for all time points after 12 h, $n=4$). Another HMG-CoA reductase inhibitor, lovastatin, also increased ecNOS protein levels in a time-, and concentration-dependent manner. Because lovastatin has a higher IC₅₀

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value for HMG-CoA reductase compared to that of simvastatin, it was 10-fold less potent in upregulating ecNOS protein levels than simvastatin at equimolar concentrations.

We have previously shown that hypoxia downregulates ecNOS protein expression (Liao, JK et al., *J Clin Invest*, 1995, 96:2661-2666). Compared to normoxia (20% O₂), exposure to hypoxia (3% O₂) resulted in a 46 ± 4 % and 75 ± 3 % reduction in ecNOS protein levels after 24 h and 48 h, respectively ($p < 0.01$, $n = 3$). In a concentration-dependent manner, treatment with simvastatin produced a progressive reversal of hypoxia-mediated downregulation of ecNOS protein levels after 48 h. At higher concentrations of simvastatin (1 and 10 mM), ecNOS protein levels were upregulated to 159 ± 13 % and 223 ± 21 % of basal levels ($p < 0.05$, $n = 3$). Co-treatment with L-mevalonic acid (400 mM) completely blocked simvastatin-induced increase in ecNOS protein levels after 48 h (35 ± 2.4 %). Treatment with L-mevalonic acid alone, however, did not produce any significant effects on basal ecNOS protein levels in untreated cells exposed to hypoxia (25 ± 3.9 %, $p > 0.05$, $n = 3$). In addition, simvastatin which was not chemically-activated had no effect on ecNOS expression. These results indicate that simvastatin-and lovastatin-mediated increases in ecNOS protein expression are mediated by inhibition of endothelial HMG-CoA reductase.

To determine whether changes in ecNOS protein levels are due to changes in ecNOS steady-state mRNA levels, we performed Northern blotting on endothelial cells exposed to normoxia and hypoxia in the presence or absence of simvastatin (1 mM) and lovastatin (10 :M). Simvastatin alone increased ecNOS mRNA levels to 340 ± 24 % ($p < 0.01$, $n = 3$). Exposure of endothelial cells to hypoxia reduced ecNOS mRNA levels by $70\% \pm 2$ % and 88 ± 4 % after 24 h and 48 h with respect to GAPDH mRNA levels, respectively. Co-treatment with simvastatin not only completely reversed hypoxia-mediated decrease in ecNOS mRNA levels, but increased ecNOS mRNA levels to 195 ± 12 % and $530 \pm 30\%$ of basal levels after 24 h and 48 h, respectively ($p < 0.01$, $n = 3$). Similarly, lovastatin (10 :M) alone increased ecNOS message to 350 ± 27 % under hypoxia and 410 ± 21 % alone ($p < 0.01$, $n = 3$). Neither simvastatin nor lovastatin caused any significant change in G-protein α and β -actin mRNA levels under normoxic or hypoxic conditions. These results indicate that the effects of HMG-CoA reductase inhibitors are relatively selective in terms of their effects on ecNOS mRNA expression.

Example 13: Effects of HMG-CoA Reductase Inhibitors on ecNOS mRNA Half-life

The half-life of ecNOS mRNA was determined in the presence of actinomycin D (5 mg/ml). Hypoxia shortened the half-life of ecNOS mRNA from 28 ± 4 h to 13 ± 3 h.

Treatment with simvastatin (1 mM) increased ecNOS half-life to 46 ± 4 h and 38 ± 4 h under
5 normoxic and hypoxic conditions, respectively ($p < 0.05$ for both, $n = 3$). These results suggest that HMG-CoA reductase inhibitors prevent hypoxia-mediated decrease in ecNOS expression by stabilizing ecNOS mRNA.

Example 14: Effects of HMG-CoA Reductase Inhibitors on ecNOS Gene Transcription

10 Nuclear run-on assays showed that hypoxia caused a $85 \pm 8\%$ decrease in ecNOS gene transcription ($p < 0.01$, $n = 3$). Treatment with simvastatin (1 mM) did not produce any significant affect on hypoxia-mediated decrease in ecNOS gene transcription ($83 \pm 6\%$ decrease in ecNOS gene transcription, $p > 0.05$ compared to hypoxia alone). Furthermore, simvastatin alone produced minimal increase in ecNOS gene transcription under normoxic
15 condition ($20 \pm 5\%$ increase in ecNOS gene transcription, $p < 0.05$ compared to normoxia control).

Preliminary studies using different amounts of radiolabeled RNA transcripts demonstrate that under our experimental conditions, hybridization was linear and nonsaturable. The density of each ecNOS band was standardized to the density of its
20 corresponding (β -tubulin band, relative intensity). To exclude the possibility that changes in (β -tubulin gene transcription are caused by hypoxia or simvastatin, another gene, GAPDH, was included on each of the nuclear run-on blots. Similar relative indices were obtained when ecNOS gene transcription was standardized to GAPDH gene transcription. The specificity of each band was determined by the lack of hybridization to the nonspecific pGEM cDNA
25 vector.

Example 15: Effect of HMG-CoA Reductase Inhibitors on Mouse Physiology

To determine whether the upregulation of ecNOS by HMG-CoA reductase inhibitors occurs in vivo, SV-129 wild-type and ecNOS knockout mice were treated with 2 mg/kg
30 simvastatin or saline subcutaneously for 14 days. The mean arterial blood pressures of wild-type and ecNOS mutant mice were as reported previously (Huang, PL et al., *Nature*, 1995, 377:239-242). The ecNOS mutants were relatively hypertensive. There was no

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significant change in mean arterial blood pressures of wild-type mice after 14 days of simvastatin treatment (81 ± 7 mmHg vs. 93 ± 10 mmHg, $p > 0.05$, $n=8$). There was also no significant group difference in heart rate, arterial blood gases and temporalis muscle temperature before ischemia or after reperfusion. Furthermore, there was no significant difference in the levels of serum cholesterol (control: 147 ± 10 vs. simvastatin 161 ± 5.2 mg/dl), creatinine and transaminases after treatment with simvastatin compared to control values.

Example 16: Effect of HMG-CoA Reductase Inhibitors on ecNOS Expression and Function in Mouse Aorta

The activity of ecNOS in the aortae of simvastatin-treated (2 mg/kg, s.c., 14 days) and saline-injected mice was determined by measuring the LNMA-inhibitable conversion of arginine to citrulline (C^{14}). The ecNOS activity in aortae from simvastatin-treated mice was significantly higher than in the control group (0.39 ± 0.09 vs. 0.18 ± 0.04 U/mg protein, $n=8$, $p < 0.05$).

The ecNOS mRNA expression in the aortae of simvastatin-treated and -untreated mice was examined by quantitative RT-PCR. There was a significantly dose-dependent 3-fold increase of ecNOS message compared to that of GAPDH in simvastatin-treated mice ($n=3$). These findings indicate that simvastatin upregulates ecNOS expression and activity in vivo.

20

Example 17: Effect of HMG-CoA Reductase Inhibitors on Cerebral Ischemia in Mice

Endothelium-derived NO protects against ischemic cerebral injury (Huang, Z et al., *J Cereb Blood Flow Metab*, 1996, 16:981-987). Therefore we examined, whether the observed upregulation of ecNOS by simvastatin in vivo has beneficial effects on cerebral infarct size. Following treatment for 14 days with 2 mg/kg of simvastatin, cerebral ischemia was produced by occluding the left middle cerebral artery for 2 hours. After 22 hours of reperfusion, mice were tested for neurological deficits using a well-established, standardized, observer-blinded protocol. The neurological motor deficit score improved in simvastatin-treated mice ($n=18$) by almost 2-fold compared to that of controls ($n=12$) (0.8 ± 0.2 vs. 1.7 ± 0.2 , $p < 0.01$).

Simvastatin-treated wild-type mice ($n=18$) had 25% smaller cerebral infarct sizes compared to untreated animals (73.8 ± 8.5 mm³ vs. 100.7 ± 7.3 mm³, $n=12$, $p < 0.05$) (Figure 4A). This effect was concentration-dependent (0.2, 2, 20 mg/kg simvastatin), persisted for up

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to 3 days, and also occurred with lovastatin treatment, albeit at higher relative concentrations. Furthermore, simvastatin increase cerebral blood flow by 23% and 35% over basal values at concentrations of 2 mg/kg and 20 mg/kg, respectively (n=8, p <0.05 for both). These findings suggest, that simvastatin decreases cerebral infarct size and neurological deficits.

5 Finally, to demonstrate that the reduction of cerebral infarct sizes by simvastatin is due to the upregulation of ecNOS, cerebral ischemia was applied to ecNOS mutant mice lacking ecNOS gene in the presence and absence of simvastatin (2 mg/kg, 14 days). There was no significant difference between the cerebral infarct sizes of simvastatin-treated and -untreated ecNOS mutant mice (n=6, p < 0.05) (Figure 4B). These findings indicate that the upregulation
10 of ecNOS mediates the beneficial effects of HMG-CoA reductase inhibitors on cerebral infarct size.

Example 18: Effect of HMG-CoA Reductase Inhibitors on ecNOS Expression in Mouse Brain

15 The ecNOS mRNA expression in the ischemic and contralateral (non-ischemic) hemispheres of mouse brain was examined by quantitative RT-PCR with respect to GAPDH mRNA levels. Simvastatin-treated mice (n=3) (2 mg/kg, 14 days) showed a 1.5-to 2-fold increase in ecNOS expression in the infarcted, ipsilateral (I) hemisphere compared to the contralateral (C), non-infarcted side. In contrast, there was no difference in ecNOS expression
20 in untreated mice between their infarcted and non-infarcted hemispheres. These findings suggest that simvastatin may reduced cerebral infarct size by selectively increasing ecNOS expression in the ischemic and hypoxic infarct zone.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

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CLAIMS

1. A method for increasing endothelial cell Nitric Oxide Synthase activity in a nonhypercholesterolemic subject who would benefit from increased endothelial cell Nitric
5 Oxide Synthase activity in a tissue comprising:
administering to a nonhypercholesterolemic subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in said tissue of the subject.
- 10 2. The method of claim 1 wherein the subject is nonhyperlipidemic.
3. The method of claim 2 wherein the amount is less than an amount which alters the blood LDL cholesterol levels in the subject by 10%.
- 15 4. The method of claim 2 wherein the amount is sufficient to increase endothelial cell Nitric Oxide Synthase activity above normal baseline levels.
5. The method of claim 2 wherein the subject has a condition comprising an abnormally low level of endothelial cell Nitric Oxide Synthase activity which is hypoxia-induced.
20
6. The method of claim 1 wherein the subject has a condition comprising an abnormally low level of endothelial cell Nitric Oxide Synthase activity which is chemically induced.
7. The method of claim 2 wherein the subject has a condition comprising an abnormally
25 low level of endothelial cell Nitric Oxide Synthase activity which is cytokine induced.
8. The method of claim 2 wherein the subject has an abnormally elevated risk of pulmonary hypertension.
- 30 9. The method of claim 2 wherein the subject has pulmonary hypertension.
10. The method of claim 2 wherein the subject has an abnormally elevated risk of an

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ischemic stroke.

11. The method of claim 2 wherein the subject has experienced an ischemic stroke.

5 12. The method of claim 2 wherein the subject is chronically exposed to hypoxic conditions.

13. The method of claims 1-12 wherein the inhibitor is selected from the group consisting of simvastatin and lovastatin.

10

14. The method of claims 1-12 further comprising co-administering an endothelial cell Nitric Oxide Synthase substrate.

15 15. The method of claims 1-12 further comprising co-administering a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity.

16. A method for increasing endothelial cell Nitric Oxide Synthase activity in a subject to treat a nonhyperlipidemic condition favorably affected by an increase in endothelial cell Nitric Oxide Synthase activity in a tissue comprising:

20 administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in said tissue of the subject.

17. The method of claim 16 wherein the nonhyperlipidemic condition is hypoxia-induced.

25

18. The method of claim 16 wherein the nonhyperlipidemic condition is pulmonary hypertension.

19. The method of claim 16 wherein the nonhyperlipidemic condition is an ischemic stroke.

30

20. The method of claim 16 wherein the nonhyperlipidemic condition is impotence.

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21. The method of claim 16 wherein the nonhyperlipidemic condition is heart failure.
22. The method of claim 16 wherein the nonhyperlipidemic condition is progressive renal disease.
- 5 23. The method of claim 16 wherein the nonhyperlipidemic condition is a gastric or esophageal motility disorder.
24. The method of claims 16-23 wherein the HMG-CoA reductase inhibitor is
10 administered in an amount which is insufficient to alter blood cholesterol levels by more than 10%.
25. The method of claim 16-23 wherein the HMG-CoA reductase inhibitor is administered acutely.
- 15 26. The method of claim 16-23 wherein the HMG-CoA reductase inhibitor is administered prophylactically.
27. The method of claim 16-23 wherein the HMG-CoA reductase inhibitor is selected
20 from the group consisting of simvastatin and lovastatin.
28. The method of claim 16-23 further comprising co-administrating a substrate of endothelial cell Nitric Oxide Synthase.
- 25 29. The method of claim 16-23 further comprising co-administering a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity.
30. A method for reducing brain injury resulting from a stroke comprising:
administering to a subject having an abnormally high risk of an ischemic
30 stroke, a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the brain tissue of the subject.

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31. The method of claim 30 wherein the HMG-CoA reductase inhibitor is administered prophylactically.
32. The method of claim 30 wherein the HMG-CoA reductase inhibitor is administered
5 acutely.
33. The method of claim 30 wherein the HMG-CoA reductase inhibitor is selected from the group consisting of simvastatin and lovastatin.
- 10 34. The method of claim 30-33 further comprising co-administering a substrate of endothelial cell Nitric Oxide Synthase.
35. The method of claims 30-33 further comprising co-administering a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity.
15
36. A method for treating pulmonary hypertension comprising:
administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the pulmonary tissue of the subject.
20
37. The method of claim 36 wherein the HMG-CoA reductase inhibitor is administered prophylactically to a subject who has an abnormally elevated risk of developing pulmonary hypertension.
- 25 38. The method of claim 36 wherein the HMG-CoA reductase inhibitor is administered to a subject who has pulmonary hypertension.
39. The method of claim 36 wherein the subject is chronically exposed to hypoxic conditions.
30
40. The method of claims 36-39 wherein the HMG-CoA reductase inhibitor is selected from the group consisting of simvastatin and lovastatin.

- 40 -

41. The method of claims 36-39 further comprising co-administering a substrate of endothelial cell Nitric Oxide Synthase.

42. The method of claims 36-39 further comprising co-administering a nonHMG-CoA
5 reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity.

43. A method for treating heart failure comprising:
administering to a subject in need of such treatment a HMG-CoA reductase
inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in
10 the heart tissue of the subject.

44. The method of claim 43 wherein the HMG-CoA reductase inhibitor is administered prophylactically.

15 45. The method of claim 43 wherein the HMG-CoA reductase inhibitor is administered acutely.

46. The method of claim 43 wherein the HMG-CoA reductase inhibitor is selected from the group consisting of simvastatin and lovastatin.

20

47. The method of claim 43 wherein the subject is nonhyperlipidemic.

48. The method of claim 43-47 further comprising co-administering a substrate of endothelial cell Nitric Oxide Synthase.

25

49. The method of claims 43-47 further comprising co-administering a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity.

50. A method for treating progressive renal disease comprising:
30 administering to a subject in need of such treatment a HMG-CoA reductase inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the kidney tissue of the subject.

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51. The method of claim 50 wherein the HMG-CoA reductase inhibitor is administered prophylactically.

52. The method of claim 50 wherein the HMG-CoA reductase inhibitor is administered
5 acutely.

53. The method of claim 50 wherein the HMG-CoA reductase inhibitor is selected from the group consisting of simvastatin and lovastatin.

10 54. The method of claim 50 wherein the subject is nonhyperlipidemic.

55. The method of claim 50-54 further comprising co-administering a substrate of endothelial cell Nitric Oxide Synthase.

15 56. The method of claims 50-54 further comprising co-administering a nonHMG-CoA reductase inhibitor agent that increases endothelial cell Nitric Oxide Synthase activity.

57. A method for increasing blood flow in a tissue of a subject comprising
administering to a subject in need of such treatment a HMG-CoA reductase
20 inhibitor in an amount effective to increase endothelial cell Nitric Oxide Synthase activity in the tissue of the subject.

58. The method of claim 57 wherein the HMG-CoA reductase inhibitor is selected from the group consisting of simvastatin and lovastatin.

25

59. The method of claim 57 wherein the subject is nonhyperlipidemic.

60. The method of claims 57-59 further comprising co-administering a substrate of endothelial cell Nitric Oxide Synthase.

30

61. The method of claims 57-59 further comprising co-administering a second agent to the subject with a condition treatable by the second agent in an amount effective to treat the

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condition, whereby the delivery of the second agent to a tissue of the subject is enhanced as a result of the increased blood flow.

62. The method of claim 60 further comprising co-administering a second agent to the
5 subject with a condition treatable by the second agent in an amount effective to treat the condition, whereby the delivery of the second agent to a tissue of the subject is enhanced as a result of the increased blood flow.

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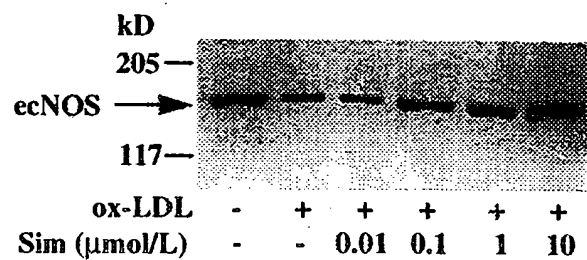


Fig. 1A

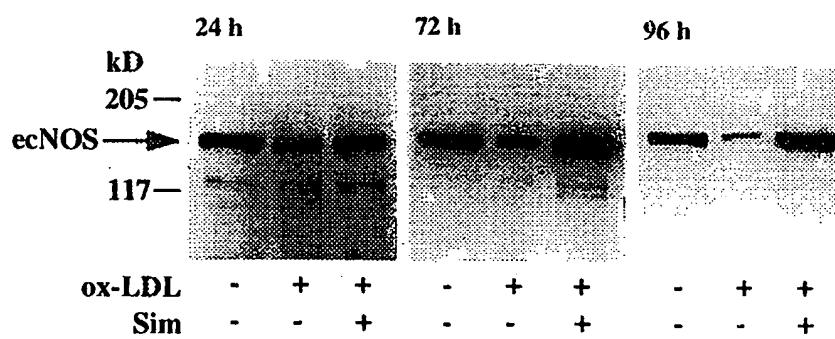


Fig. 1B

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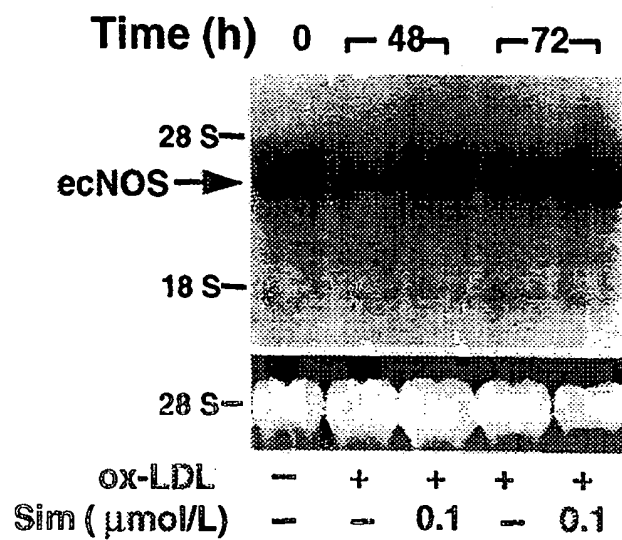


Fig. 2A

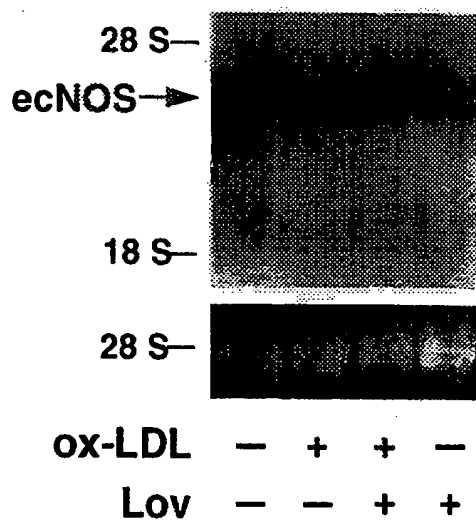


Fig. 2B

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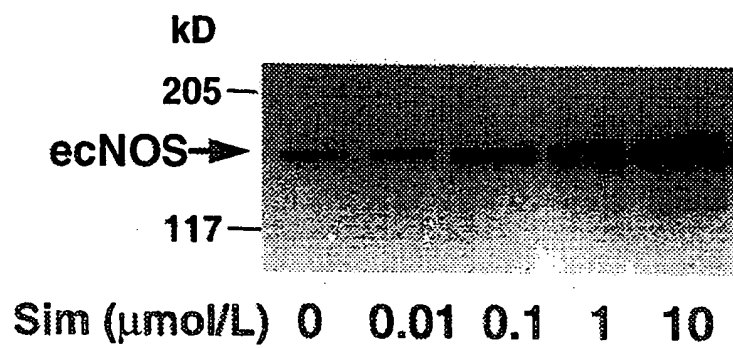


Fig. 3A

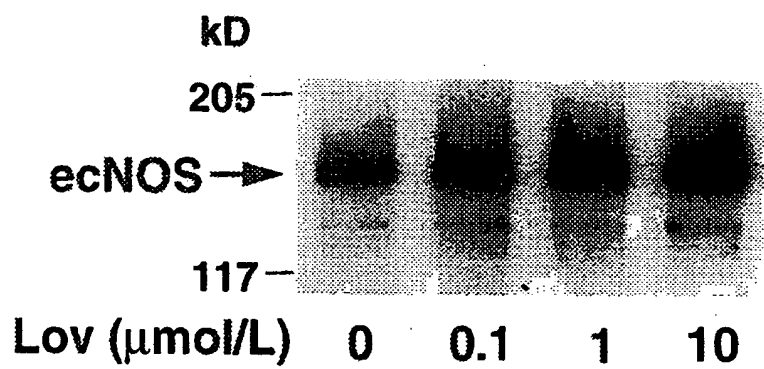


Fig. 3B

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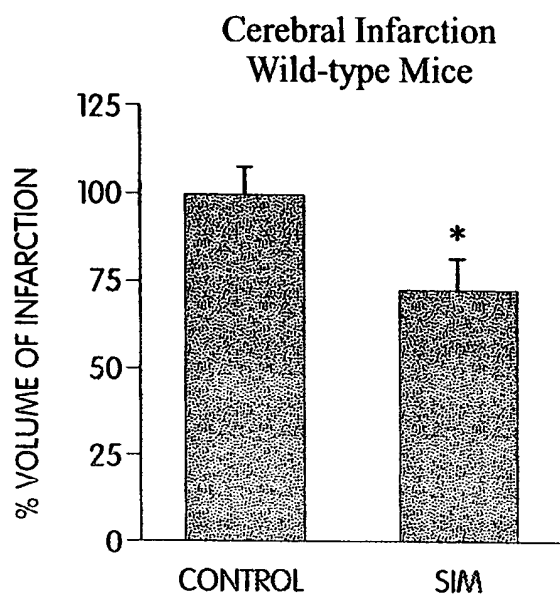


Fig. 4A

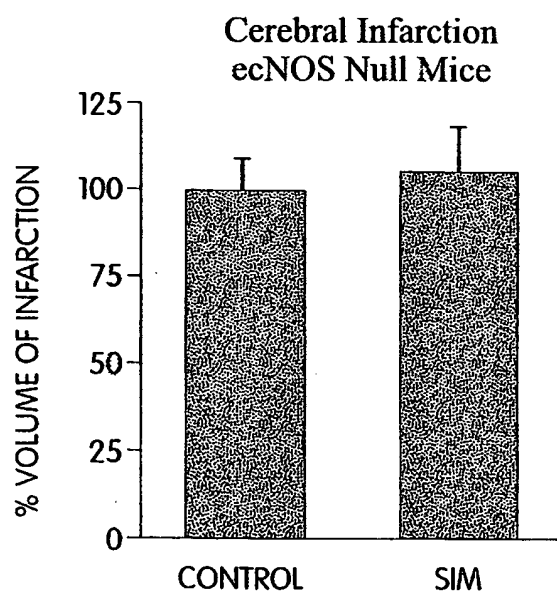


Fig. 4B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21464

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	O'DRISCOLL ET AL: "the HMG-CoA reductase inhibitor simvastatin improves endothelial function within one month" CIRCULATION, vol. 94, no. 8, 1996, page 1-401 XP002091894 see abstract	1-62
X	EP 0 671 171 A (SQUIBB BRISTOL MYERS CO) 13 September 1995 see page 10 see claim 2	30-35, 43-49

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

2 February 1999

Date of mailing of the international search report

16/02/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

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Trifilieff-Riolo, S

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21464

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
P,X	ENDRES ET AL: "stroke protection by HMG-CoA reductase inhibitors mediated by endothelial nitric oxide synthase" PROC NATL ACAD SCI USA, vol. 95, no. 15, 21 June 1998, pages 8880-8885, XP002091890 see abstract see page 8884 "discussion" to page 8885 ----	1-4,10, 11,13, 16,19, 24-27, 30-33, 57-59
P,X	ENDRES ET AL: "simvastatin pretreatment protects from focal cerebral ischemia" STROKE, vol. 29, no. 1, January 1998, page 325 XP002091891 see abstract ----	1-4,10, 11,13, 16,19, 24-27, 30-33, 57-59
P,X	DI NAPOLI ET AL: "simvastatin exerts a direct anti-ischemic effect" EUR. HEART J., vol. 19, August 1998, page 124 XP002091892 see abstract ----	1-4,10, 13,16, 21, 24-27, 43-47
P,X	LAUFS ET AL: "inhibition of HMG-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase" J BIOL CHEM, vol. 272, no. 50, 12 December 1997, pages 31725-31729, XP002091893 see abstract see page 31728 - page 31729 ----	1-9,12, 13, 16-18, 24-27, 36-40
P,X	WO 98 34626 A (UNIV SOUTH CAROLINA ;SINGH INDERJIT (US)) 13 August 1998 see page 8, line 7 see page 13, line 1 - line 13 see page 13, line 23 - line 30 -----	1-5,16, 17,22, 24,26, 27,50, 51,53,54
P,X		14,28,55

INTERNATIONAL SEARCH REPORT

I. national application No.

PCT/US 98/21464

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-62
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
In view of the large number of compounds which are defined by the wording
of the claims, the search has been performed on the general idea and
compounds mentioned in the examples of the description.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/21464

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0671171 A	13-09-1995	AU 1022795 A	27-07-1995
		CA 2140498 A	19-07-1995
		JP 7206712 A	08-08-1995
		US 5691375 A	25-11-1997
		US 5674893 A	07-10-1997
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WO 9834626 A	13-08-1998	AU 6533898 A	26-08-1998
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